

Stimulation of Myoblast Membrane Protein Synthesis by 25-Hydroxy-Vitamin D₃*

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The effects of 25-hydroxy-vitamin D₃ (25OHD₃) on myoblast protein synthesis were studied in connection with its role on muscle cell phosphate metabolism. The sterol markedly increased leucine incorporation into total cell proteins in cultured chick embryo myoblasts. This enhancement was greater than that produced by 1,25-dihydroxy-vitamin D₃ (1,25(OH)₂D₃) and occurred prior to a significant stimulation of cell phosphate accumulation. Maximum effects of 25OHD₃ (8 h) on myoblast phosphate uptake were suppressed by cycloheximide indicating that they are mediated by *de novo* protein synthesis. At a similar treatment period, labelling of myoblasts with [³H]leucine (control) and [¹⁴C]leucine (+ 25OHD₃) followed by co-electrophoresis of total protein extracts on SDS-PAGE and isoelectrofocusing gels revealed that the sterol selectively affects the synthesis of proteins of 20 kDa and 50 kDa. These macromolecules were recovered in the microsomal fraction after differential centrifugation of homogenates. Further fractionation of myoblast microsomes on sucrose density gradients showed co-localization of the 50 kDa and 20 kDa proteins with microsomal subfractions which preferentially bind [³H-α]bungarotoxin, suggesting that the proteins induced by 25OHD₃ are associated to plasma membranes and may play a role in the effects of the sterol on cell phosphate uptake.

Introduction

Vitamin D plays an essential role in the mechanisms of phosphorus homeostasis in higher animals. The sterol stimulates the absorption of phosphate by the intestine [1–4], the mobilization of phosphate from bone [5] and enhances the reabsorption of phosphate by the renal tubule [6–8]. The hormonally active form of vitamin D₃, 1,25-dihydroxy-vitamin D₃ (1,25(OH)₂D₃), has been implied in these effects [9–11]. However, 25-hydroxy-vitamin D₃ (25OHD₃), the most abundant vitamin D metabolite in plasma, has also been shown to affect phosphate fluxes in intestine *in vitro* [12, 13] and kidney *in vivo* [7].

There is evidence which indicates that muscle may be another tissue which participates in the maintenance of plasma phosphate [14]. Moreover, there are data which suggest that vitamin D regulates phosphate exchange between skeletal muscle and extracellular fluid. It has been reported [15] that 25OHD₃, but not 1,25(OH)₂D₃, affects *in vivo* phos-

phate accumulation by rat muscle. Similar observations have been made in chick skeletal muscle *in vitro* [16]. 25OHD₃ also stimulated phosphate uptake by cultured chick embryonic muscle cells. Evidence was obtained in these studies which suggested that the action of 25OHD₃ on muscle cell phosphate metabolism is localized on plasma membranes and requires new protein synthesis [16]. To study the possibility that 25OHD₃ affects the synthesis of myoblast membrane proteins which may mediate its action on phosphate uptake, we have characterized the effect of the sterol on myoblast protein synthesis by double labelling procedures.

Materials and Methods

Materials

Purified samples of chemically synthesized 1,25(OH)₂D₃ and 25OHD₃ were supplied by Hoffman-La Roche (Nutley, N. Y., U.S.A.) and The Upjohn Co. (Kalamazoo, Mich., U.S.A.), respectively. ³²P-Na₂HPO₄ was purchased from the Atomic Energy Commission (Argentina). [3,4,5-³H-(N)]L-leucine and [1-¹⁴C]L-leucine were obtained from New England Nuclear Co. (Boston, Mass., U.S.A.). Alpha-bungarotoxin, N(propionyl) ³H- propionylated was supplied by Amersham (Buckinghamshire, England). Bovine pancreas trypsin, type III-s

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(10,000–13,000 U/mg protein) and protein molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Other reagents used in this study were of analytical grade.

Methods

Chick embryo myoblast cultures were prepared as previously described [16–18]. Fibroblast-free cells were cultured 24 h in Eagle's Minimum Essential Medium (MEM) containing 10% (v/v) chick serum, 10% (v/v) chick embryo extract, streptomycin (0.20 mg/ml), penicillin (0.24 mg/ml), and nystatin (0.015 mg/ml). The medium was then replaced by MEM containing 10% chick serum, 2% chick embryo extract and antibiotic-antimycotic solution (8:1:0.2), and the cells were cultured for different times up to 24 h after the addition of vitamin D₃ metabolites. The sterols were added dissolved in ethanol. Ethanol alone was added to control cultures. The concentration of solvent was not greater than 0.1%. Concentrations and treatment intervals with metabolites are indicated for each experiment in the Results section. Viability of the cells from control and treated preparations was about 90%, as determined by measurement of trypan blue exclusion [19].

For subcellular fractionation, control and 25OHD₃ treated (8 h, 40 mg/ml) cells were washed four times with 0.9% ClNa and homogenized for 15 sec in an Ultra-Turrax homogenizer at medium speed in 0.2 M sucrose, 0.1 M ClK, 20 mM Tris-HCl (pH 7.4), 0.3 mM phenyl-methylsulfonylfluoride (pmsf). These operations were performed in the cold room. Heavy fraction (containing nuclei and myofibrils), mitochondria and microsomes were isolated by centrifugations of homogenates at 600 × g (10 min), 10,000 × g (15 min), and 150,000 × g (60 min), respectively. The final supernatant corresponds to the soluble fraction.

Plasma membrane-enriched myoblast fractions were obtained essentially as previously described [20]. The cells from control and sterol-treated cultures were homogenized by fifteen up-and-down strokes in a glass homogenizer, using a buffer containing 0.25 M sucrose (8.3%), 1 mM Tris-HCl (pH 7.4), 0.3 mM pmsf. Nuclei, myofibrils and mitochondria were collected by centrifugation at 1700 × g (10 min). Microsomes were then sedimented by centrifugation of the supernatant at 33,000 × g (60 min). The resulting pellet was sus-

pended in 1 ml of the same buffer and layered over a discontinuous sucrose density gradient formed by adding 0.2 ml of 55% (w/w) sucrose, then 1 ml each of 40%, 32%, 27% and 20% sucrose. The samples were centrifuged in a SW 50.1 rotor at 206,000 × g for 45 min. Microsomal subfractions comprising a sucrose concentration range (%) of 8.3–20 (I), 20–27 (II), 27–32 (III), 32–40 (IV) and 40–55 (V), were collected.

Myoblast phosphate uptake was measured as previously described [16]. Briefly, cells cultured in absence or presence of 25OHD₃ were washed with 0.2% glucose – Krebs-Henseleit solution (pH 7.4) containing 1 mM phosphate. The cells were then incubated in the same solution labelled with [³²P]phosphate (0.1 μCi/ml) at 37 °C under 95% O₂–5% CO₂. After 5 min or 60 min of incubation, aliquots of 1 ml were diluted with 30 ml of cold Krebs-Henseleit solution and centrifuged 1 min at 1200 × g. The cells were homogenized in 0.1% sodium dodecyl sulfate (SDS). Protein content was assayed by the procedure of Lowry [21] and radioactivity was measured using a Beckman liquid scintillation spectrometer.

For [³H-alpha]bungarotoxin binding measurements, myoblasts cultured 24 h in medium 8:1:1 followed by 8 h in medium 8:1:0.2 were incubated in MEM containing [³H-alpha]bungarotoxin (9.7×10^{-3} μCi/ml) under 95% O₂–5% CO₂ for 10 min at 37 °C. The binding was stopped by centrifuging 1 min at 1200 × g. The cellular pellet was washed four times with 0.9% ClNa, 2 mg/ml BSA and twice with 0.9% ClNa, homogenized and centrifuged to obtain microsomal subfractions, as described above. Radioactivity was measured in the total homogenate and in each microsomal subfraction.

The measurement of radioactive leucine incorporation (0.01 μCi/ml) into myoblast total proteins was carried out during 2 h in 0.2% glucose – Krebs-Henseleit solution at 37 °C under 95% O₂–5% CO₂. Cells were centrifuged for 1 min at 1200 × g and washed twice with cold Krebs-Henseleit solution. Proteins were precipitated with 10% TCA and dissolved in 0.5 N NaOH. Radioactivity and protein content [21] were measured.

For double-labelling analysis, control and 25OHD₃-treated myoblasts were incubated with [³H]- or [¹⁴C]leucine, respectively, as above. Cells were washed, homogenized and the homogenates centrifuged to obtain subcellular fractions, as indicated before. Homogenate or subcellular fraction

proteins were precipitated with 10% trichloroacetic acid followed by solubilization in electrophoresis buffer containing 5% (w/v) SDS, 36% (w/v) sucrose, 0.33% (v/v) beta-mercaptoethanol, 0.67% (w/v) bromophenol blue, 0.33 M Tris-bicine. Equal amounts of proteins from control and sterol-treated samples were mixed and electrophoresed on 10% SDS-polyacrylamide gels, as previously described [22]. Gels were sliced in 2 mm fractions. Fractions were dissolved in 100% H_2O_2 at 60–70 °C. $^{14}C/^{3}H$ ratios were determined for each fraction and plotted as a function of the relative mobility. The following molecular weight standards (kDa) were used: bovine serum albumin (66); ovalbumin (45); pepsin (34.7); trypsinogen (24); beta-lactoglobulin (18.4); lysozyme (14.3); cytochrome *c* (12.4).

For isoelectrofocusing analysis, double-labelled proteins of myoblast homogenates were included in 7.7% polyacrylamide gels and separated in a 2–10 pH unit gradient at 4 °C. $^{14}C/^{3}H$ ratios were determined in 3 mm slices of sample gels. Gel slices from parallel runs were extracted with water and the pH measured. Hemoglobin (pI 6.8) and ovalbumin (pI 4.6) were used as standards.

Results

It has been previously shown that physiological concentrations of 25OHD₃ stimulate phosphate uptake by cultured embryonic muscle cells [16]. The data of Fig. 1 show that this effect is preceded by a marked increase in leucine incorporation into myoblast proteins. Similar doses (40 mg/ml) or physiological levels (0.05 mg/ml) of 1,25(OH)₂D₃ were less effective (Fig. 1).

As shown in Fig. 2, 25OHD₃ stimulated both the velocity and total capacity of phosphate accumulation by myoblasts (measured at 5 min and 60 min of uptake, respectively, according to previous studies [16]), indicating that the action of the sterol is related at least in part to an effect on phosphate entry into myoblasts and not only due to changes in cell phosphate organization.

Cycloheximide abolished the increase in total cell phosphate accumulation induced by 25OHD₃ as shown before [16] and also the stimulation of phosphate uptake velocity by the metabolite, without significantly affecting control cultures (Fig. 2).

In order to investigate whether 25OHD₃ selectively affects protein synthesis, double-labelling analysis

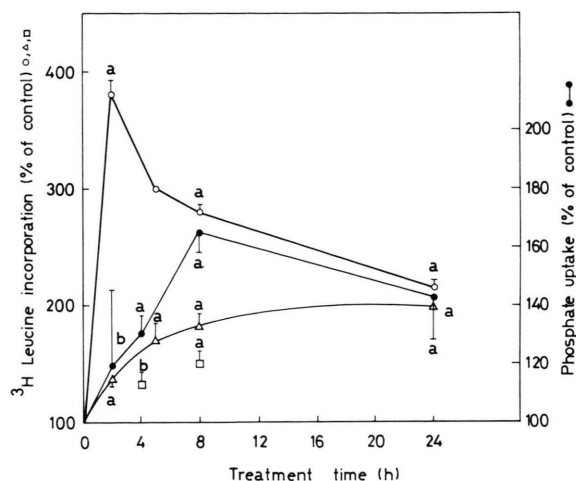


Fig. 1. Time course of effects of vitamin D₃ metabolites on myoblast protein synthesis and phosphate accumulation. Primary cultures of chick embryo myoblasts were incubated in the absence or presence of 25OHD₃ (40 mg/ml, ○, ●) or 1,25(OH)₂D₃ (40 mg/ml, △; or 0.05 mg/ml, □) for 2–24 h at 37 °C. [3H]leucine incorporated into cell proteins and [^{32}P]phosphate accumulated by myoblasts during 60 min were measured as indicated in Methods. Means \pm SD are given, $n = 4$. (a) $p < 0.0005$; (b) $p < 0.25$.

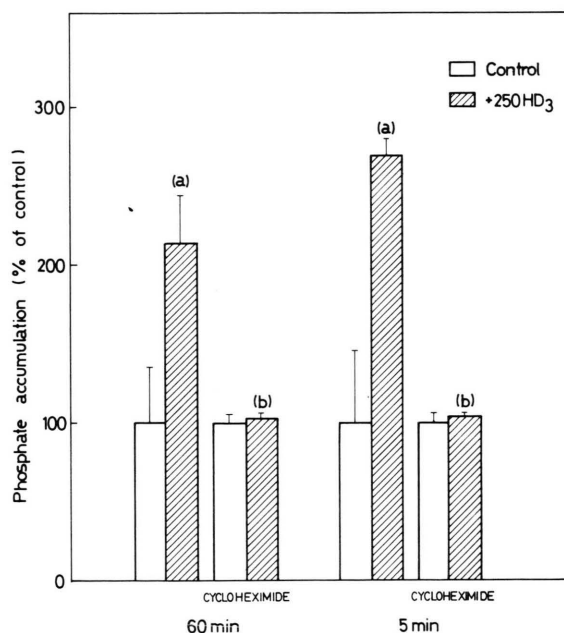


Fig. 2. Suppression by cycloheximide of 25-hydroxy-vitamin D₃-dependent myoblast phosphate accumulation. Myoblasts were treated for 8 h with physiological concentrations of 25OHD₃ in the absence or presence of 50 μ M cycloheximide. [^{32}P]phosphate accumulated by cells during 60 min and 5 min was measured as indicated in Methods. Means \pm SD are given, $n = 4$. (a) $p < 0.0025$; (b) n.s.

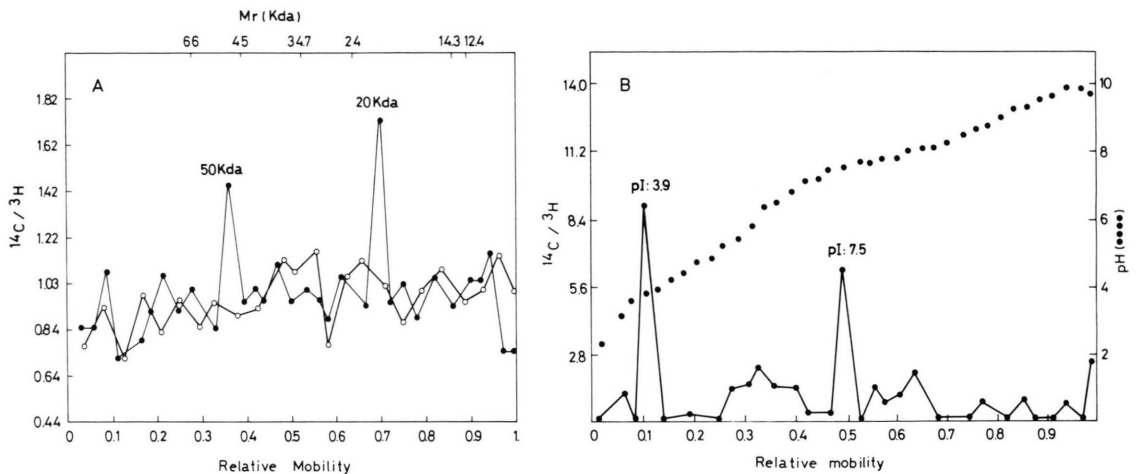


Fig. 3. Differential labelling of myoblast proteins in response to 25-hydroxy-vitamin D₃. Control and 25OHD₃-treated (8 h, 40 mg/ml) myoblasts were labelled with [³H]leucine or [¹⁴C]leucine, respectively. Total cell proteins from control and treated cultures were mixed and separated by either SDS-PAGE (A) or isoelectrofocusing (B). ¹⁴C/³H ratios were determined in each gel fraction as indicated in Methods. Parallel double-labelling control experiments were carried out using untreated cells. ○—○ Control (¹⁴C)/control (³H); ●—● 25OHD₃ (¹⁴C)/control (³H). Representative profiles of five (A) and three (B) experiments are given.

were performed, as described in Methods. The separation of double-labelled total myoblast proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that 25OHD₃ stimulates the synthesis of at least two proteins corresponding to molecular masses of 2 kDa and 50 kDa (Fig. 3A). Isoelectricfocusing separation of the same samples of labelled myoblast proteins also evidenced induction of two proteins in response to the sterol, having isoelectric points of 3.9 and 7.5 (Fig. 3B). These changes could be reproduced consistently in several experiments. On the basis of the relative heights of double-labelled peaks obtained by both electrophoretic methods, it is likely that isoelectric pH values of 3.9 and 7.5 correspond to the 20 kDa and 50 kDa proteins, respectively.

Similar double-labelling analysis of myoblast subcellular fractions showed that the 25OHD₃-dependent proteins are associated to the microsomal fraction (Fig. 4). Further purification of myoblast

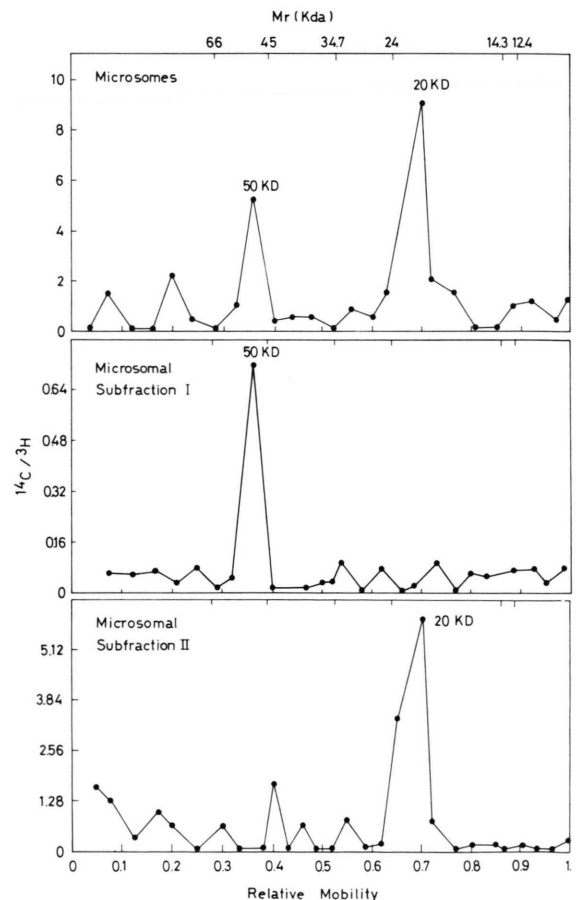


Fig. 4. Subcellular localization of 25-hydroxy-vitamin D₃-dependent myoblast 50 kDa and 20 kDa proteins. Control and sterol-treated (8 h, 40 mg/ml) myoblasts were labelled with [³H]leucine or [¹⁴C]leucine, respectively. Microsomes (33,000 × g, 60 min) were further fractionated on a discontinuous 8.3–55% sucrose density gradient at 206,000 × g for 45 min. ¹⁴C- and ³H-labelled proteins from microsomes or each gradient fraction were mixed and separated by SDS-PAGE. ¹⁴C/³H ratios were determined in gel slices. A representative profile of three experiments is given.

Table I. [^3H -alpha]Bungarotoxin-binding activity of myoblast microsomal subfractions. Myoblast (300–400,000 cell/ml) were incubated with [^3H -alpha]bungarotoxin (9.7×10^{-3} $\mu\text{Ci/ml}$) for 10 min at 37 °C. Microsomal subfractions were then isolated on 8.3–55% discontinuous sucrose density gradients, as in Fig. 4 and the radioactivity determined.

	[^3H -alpha]- Bungarotoxin-bound [cpm/mg protein]	% Sucrose
Total homogenate	27.53	—
Microsomal subfraction		
I	500.00	8.3–20
II	1076.90	20 –27
III	36.40	27 –40
IV	266.70	40 –45
V	190.50	45 –55

microsomes on discontinuous sucrose density gradients showed co-localization of the 50 kDa and 20 kDa proteins with microsomal subfractions I (8.3–20% sucrose) and II (20–27% sucrose), respectively (Fig. 4). These subfractions bound preferentially [^3H -alpha]bungarotoxin (Table I), suggesting thereby that they are enriched in sarcolemma membranes.

Discussion

The presence of a phosphate transport system mediated by the cell membrane sodium gradient in chick myoblasts with characteristics similar to those reported for the phosphate carrier of intestine and kidney has been shown [16]. Studies both *in vivo* and *in vitro* have demonstrated that 25OHD₃ stimulates muscle cell phosphate accumulation [15, 16]. Evidence was obtained suggesting that the metabolite affects Na⁺-dependent phosphate transport across the muscle plasma membrane [16, 23]. In agreement with these results, we have now observed increased phosphate uptake velocity by myoblasts in response to 25OHD₃ (Fig. 2).

Previous studies have shown that the mode of action of 25OHD₃ is not related to changes in myo-

blast phospholipid and fatty acid composition [24]. The present investigation implies that modifications in protein synthesis could be involved in the stimulation of phosphate transport by the sterol. This was suggested by the observation that 25OHD₃ increased leucine incorporation into proteins prior to a significant stimulation of phosphated uptake. The effects of the sterol on protein synthesis cannot be ascribed to its conversion into 1,25(OH)₂D₃ as the latter metabolite was less active. This is further supported by the observation of Birge *et al.* [15] showing that administration of 25OHD₃ to nephrectomized rats increases leucine incorporation into muscle proteins. In agreement with these results, cycloheximide blocked the responses of increased velocity and total myoblast phosphate uptake, indicating that they are mediated by *de novo* protein synthesis (Fig. 2).

25OHD₃ appeared to selectively affect the synthesis of only two proteins (20 kDa and 50 kDa). Moreover evidence was obtained suggesting that these macromolecules are localized in sarcolemma membranes (Fig. 3, 4 and Table I).

The effect of 25OHD₃ on myoblast protein synthesis is evidently specific, since 1,25(OH)₂D₃ has been shown to induce a different protein labelling profile [25]. This is in line with recent evidence indicating that 25OHD₃ may differ from 1,25(OH)₂D₃ in its effects on cell metabolism [17, 26]. There is no information on the mechanism by which 25OHD₃ induces the synthesis of myoblast membrane proteins. Only the presence of a receptor for 1,25(OH)₂D₃ has been detected in myoblasts [27, 28]. Future studies should address this issue and investigate the role of the proteins of 20 kDa and 50 kDa in phosphate uptake by muscle cells.

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